Wild Strains of *Agaricus bisporus*: a Source of Tolerance to Dry Bubble Disease

M. L. Largeteau; J. P. P. Baars; S. Juarez del Carmen;
C. Regnault Roger; J. M. Savoie

Institut National de la Recherche Agronomique (INRA), BP81, F-33883 Villenave d'Ornon, France.
Applied Plant Research, Mushroom Research Unit, P.O. Box 6042, NL-5960 AA Horst, The Netherlands.
UFR Sciences et Techniques, Univ. Pau et Pays de l'Adour, F-64012 Pau Université cedex, France

The button mushroom *Agaricus bisporus* is susceptible to various pests and diseases. Dry bubble, caused by the Hyphomycete *Verticillium fungicola*, is currently the more serious disease and is worldwide in distribution. Cultivars are susceptible and the pathogen develops resistance towards the very few fungicides admitted at mushroom farms. Breeding for resistance is necessary and wild strains of *A. bisporus* are putative sources of tolerance to *V. fungicola*. We present results on the susceptibility of some wild strains of the INRA-CTC collection and the PPO MRU collection. Besides the severity of the disease, the strains were also compared for their ability to develop each of the symptoms induced by the pathogen: spotted mushrooms, stipe blow-out and spheroid masses (the bubbles) which are the typical symptom of the disease.

***Agaricus bisporus* 2100, cultivated in numerous French mushroom farms, was used to assess the aggressiveness of various isolates of *V. fungicola* var. *fungicola*, the variety responsible for the disease in Europe at present. Significant variability in aggressiveness was observed. Isolate VCTC, which induced severe symptoms on *A. bisporus* 2100 (30-40% of diseased mushrooms), revealed interesting tolerance (10-18% of diseased mushrooms) among five wild *A. bisporus* strains and hybrids between wild strains. A cross test was performed with two cultivars and seven wild stains of *A. bisporus* contaminated with five *V. fungicola* isolates, two of var. *fungicola* and three of var. *aleophilum*, the latter identified as responsible for the disease in USA and Canada. The wild strains screened in this experiment were far more tolerant than the cultivars, exhibiting 3-9% of diseased mushrooms compared to 20-22%. All the strains were more susceptible to the pathogens of var. *aleophilum* than to those of var. *fungicola*.**

These experiments showed that very tolerant material exists in collection and can be used as parents to breed for resistance. The greater susceptibility of *A. bisporus* to *V. fungicola* var. a*leophilum* must be taken into consideration in breeding programmes, this variety being present in North America and being isolated in Europe in the past.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
 ISBN 84-9769-107-5

1. Introduction

The INRA and the PPO MRU collections of *Agaricus bisporus* both consist of wild strains of a large genetic diversity and a great variability in phenotypic traits such as colour (white to dark brown) and morphology. The PPO MRU collection is derived from the collection of the Agaricus Resource Program (ARP program, Kerrigan, 1996). At the time when chemicals become more and more restricted breeding for *A. bisporus* strains that are resistant to the major diseases, especially dry bubble, is of prime importance. Resistant (or very tolerant) wild strains need to be selected and introduced in crosses. This paper describes the choice of isolates of *Verticillium fungicola* allowing the identification of strains potentially tolerant to any isolate of the pathogen, and shows the possibility to find some tolerant *A. bisporus* strains during the screening of collections.

2. Material and Method

2.1. *Agaricus bisporus*

Commercial strains 2100 (Amycel, France), A15 (Sylvan, the Netherlands), U1 (Horst, NL), wild strains W1, W2, W3, W4, W5, W7, W8 (PPO MRU collection), WA, WB (INRA collection) and hybrids HW1, HW3, HW4 (obtained from wild strains, INRA collection) were screened for susceptibility.

2.2. *Verticillium fungicola*

Isolates of *V. fungicola* var. *fungicola* collected from various geographical origins and of different dates of collection, and var. *aleophilum* isolates are representative of the clonal population of *V. fungicola* responsible for recent outbreak in Pennsylvania (Collopy *et al*. 2001) (Table 1).

Table 1
Isolates of *Verticillium fungicola* screened for aggressiveness

Isolate variety	*Isolate code*	*Date*	*Geographic origin*	*Source*
fungicola	VCF	1996	Chancelade, France	INRA-Bx
fungicola	VCTC	1997	St Paterne, France	INRA-Bx
fungicola	VF	1987	St Paterne, France	INRA-Bx
fungicola	VK	1994	Nancy, France	INRA-Bx
fungicola	VMX1	2002	Xalapa, Mexico	INRA-Bx
fungicola	V9503	1995	The Netherlands	PPO MRU
fungicola	V010404	2001	Limburg, The Netherlands	PPO MRU
aleophilum	V02	1999	Chester County, PA, USA	Collopy *et al.* (2001)
aleophilum	V07	1999	Chester County, PA, USA	Collopy *et al.* (2001)
aleophilum	V17	1999	Chester County, PA, USA	Collopy *et al.* (2001)

2.3. *Pathogenicity test in culture*

INRA tests: *Agaricus bisporus* was grown in 0.9 m^2 trays filled with commercial compost spawned at 0.8%. No fungicide was added, and cultures were covered with plastic film. Spawn-run took place for 13 days in a climatic room set at 24°C, 92% relative humidity before casing (85% limestone and 15% peat, v:v, not treated with fungicide) was applied. Nine days after casing the room temperature was decreased to 16°C. Eleven days after casing the cultures were ruffled and a conidial suspension of *V. fungicola* var. *fungicola* was sprayed onto the surface of the casing layer at a rate of 10^6 conidia / m^2. Each *V. fungicola* isolate was supplied to six trays. Bait cultures made of six uninfected trays were used to assess contamination caused by spores produced by affected mushrooms. The experiment was performed twice with two different batches of compost. Healthy mushrooms, spotted mushrooms (S), stipe blow-out (BO) and bubbles (DB) were harvested for 4 weeks and weighted separately. Data reported were means from the two cell tests. A first test compared the level of susceptibility of *A. bisporus* Amycel 2100 to six isolates of *V. fungicola*, VCF, VCTC, VF, VK, VMX1 and V95. In a second test, the isolate VCTC was used to assess the susceptibility of two wild strains (WA and WB) and three hybrids between wild strains

(HW1, HW3 and HW4). Bait cultures consisted in six crates of uninoculated WB.

MRU-test: *Agaricus bisporus* A15, U1, W1-5, W7 and W8 were cultivated in crates containing 15 kg of compost. After spawning, malathion was sprayed onto the compost. Spawn-run took place at 24°C. After one day, the cultures were covered with paper. After 17 days, casing soil (CNC, standard composition, not treated with fungicide) was applied. Subsequently, the cultures were infected with *V. fungicola* V9503, V010404, V02, V07 and V17 by pouring 100 mL of a suspension of freshly harvested conidia (1.5×10^6 spores / m^2) on top of the casing soil. Eleven days after casing the cultures were ruffled. Fourteen days after casing, the temperature was decreased down to 17°C. Cell tests, each with one crate per *A. bisporus*-V. *fungicola* combination and one control crate (uninfected) per *A. bisporus* strain, were repeated three times. Harvested mushrooms were divided into three categories: healthy, spotted and affected (stipe blow-out and bubbles) mushrooms and counted.

2.4. *Data analyses*

Data reported were percentages of the total crop but statistical analyses were performed on arc sinus transformed data using the general linear model provided by the SAS system (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. *Factors of variation in the pathogenicity test of* Agaricus bisporus *strains*

Significant cell-test effects were detected (Table 2) which corroborated the observations of Sonnenberg *et al.* (2005) who reported that the infection levels of *A. bisporus* vary considerably from crop to crop for unknown reasons. Previous experiments carried out both at INRA facilities and PPO MRU facilities with a single strain of *A. bisporus* contaminated with a single isolate of *V. fungicola* have shown the homogeneity of the climatic cell (data not shown). We postulated that uncontrolled variations between batches of compost were responsible for the variations in susceptibility observed from crop

to crop. The absence of significant interaction between the cell tests and the treatments (Table 2) means that the classification of the isolates of *V. fungicola* for aggressiveness was the same in the two cell tests and consequently allowed us to consider mean values from the two tests.

Table 2
Analysis of variance for the various symptoms (INRA test 1).

Variable[1]	*Source*	*df*	*Mean square*	*F value*
S	Cell test	1	35.1736	3.59 ns
	Treatment	6	231.3690	23.58 **
	Cell test*Treatment[2]	6	15.7446	1.60 ns
	Crate	5	22.9408	2.34 ns
BO + DB	Cell test	1	1123.2729	71.08 **
	Treatment	6	1237.2523	78.29 **
	Cell test*Treatment	6	14.3596	0.91 ns
	Crate	5	8.3585	0.53 ns
S + BO + DB	Cell test	1	430.2089	31.39 **
	Treatment	6	1336.4944	97.50 **
	Cell test*Treatment	6	24.8501	1.81 ns
	Crate	5	21.5202	1.57 ns

1. S = spot, BO = stipe blow-out and DB = dry bubble. 2. *V. fungicola* or control (bait culture).
** = significant at $P < 0.01$, * = significant at $P < 0.05$ and ns = not significant at $P = 0.05$.

The disease levels of *A. bisporus* 2100 varied dramatically with the isolate of *V. fungicola* supplied. Sporophores with stipe blow-out were more abundant on crates inoculated with VCF, the less aggressive isolate, than on control crates used as bait culture, but the disease level did not differ significantly between both treatments. The percentage cumulating mushrooms with stipe blow-out and bubbles was nine times higher after inoculation of VCTC, V95 and VMX1 than after the supply of VCF. Differences observed for spotted mushrooms were also significant but of far less magnitude (Table 3). A significant correlation ($P< 0.01$) was found between the percentage of spotted mushrooms and that of stipe blow-out whereas none was detected between the percentage of these symptoms (S + BO) and that of bubbles.

The various symptoms depend on the stage of development of the mushroom at the time of infection (van de Geijn, 1982; North and Wuest, 1993; Rinker and Wuest, 1994). But as this cell-test was performed with a single strain of *A. bisporus* the propension in developing rather bubbles or rather other symptoms should be related to the pathogen. VF induced as numerous stipe blow-out and spotted mushrooms but far less bubbles than the aggressive isolates VMX1 and VCTC. Contamination by spores produced by bubbles cannot explain these differences because of the low symptom levels observed on bait cultures and crates contaminated with VCF.

Total mushroom production (including both diseased and healthy mushrooms), expressed as g/kg substrate, did not vary significantly with the treatment and was not significantly different from that observed for the control (Table 4).

Table 3
Response of *Agaricus bisporus* 2100 to various isolates of *Verticillium fungicola*

Treatment	*Percentages of*				
	S	*BO*	*DB*	*BO + DB*	*S + BO + DB*
VMX1	11.4 ab[1]	4.3 ab	20.9 a	25.2 a	36.60 a
VCTC	13.0 ab	4.7 ab	20.0 a	24.7 a	37.80 a
V9503	14.3 a	6.9 a	15.6 a	22.5 a	37.20 a
VF	12.6 ab	5.6 a	6.0 b	11.6 b	24.30 b
VK	10.1 b	3.1 bc	3.6 c	6.7 c	16.89 c
VCF	7.4 c	2.1 c	0.7 d	2.8 d	10.20 d
Control	2.9 d	0.7 d	0.6 d	1.3 d	4.20 d

1. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

Table 4
Effect of contamination with *Verticillium fungicola* on the total crop

Treatment	*Total crop*	*(g/kg substrate)*
VMX1	261.0	ab[1]
VCTC	285.1	a
V9503	262.1	ab
VF	282.5	a
VK	285.4	a
VCF	289.8	a
Control	281.1	a

1. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

The aggressiveness of the isolates of *V. fungicola* was not correlated with the date in collection ($r^2 = 0.21$ for BO + DB and 0.03 for S, df = 4). In the same culture conditions, the isolate CBS440.34, in collection since 1934, was not affected by long time storage and gave 38.2% of affected mushrooms including 22.9% of bubbles.

3.2. *Variability in susceptibility of* Agaricus bisporus *strains to* Verticillium fungicola

For the purposes of breeding a Verticillium resistant strain, an Agaricus strain identified as resistant or tolerant to *V. fungicola* in cell-tests must also be resistant or tolerant to any Verticillium isolate. To identify material for breeding programmes and to characterise hybrids we have chosen the aggressive isolate VCTC. In other cell-tests this isolate was as aggressive towards *A. bisporus* Euromycel 31 as towards 2100 (Largeteau *et al.*, 2004). Despite its high aggressiveness VCTC revealed significant differences in susceptibility within a group of wild strains and hybrids (Table 5).

As observed for *A. bisporus* 2100, the inoculation of *V. fungicola* had no significant influence ($P = 0.05$) on the total crop of WB; 1871 g and 1941 g $crate^{-1}$ were harvested on inoculated crates and bait cultures, respectively.

Other cell-tests are in progress to characterize the INRA collection of *A. bisporus* and have identified some wild strains of substantial yield and high tolerance to VCTC (not shown). The large scale screening of wild strains for susceptibility to *V. fungicola* performed by Sonnenberg *et al.* (2005) identified several strains showing less than 5% affected mushrooms after three flushes.

Table 5
Comparison of wild strains and hybrids for their susceptibility to *Verticillium fungicola* VCTC

A. bisporus	*Percentages of*				
	S	*BO*	*DB*	*BO + DB*	*S + BO + DB*
WA	1.1 b	7.5 a	6.2 a	13.6 a	14.8 ab
WB	8.5 a	4.1 ab	5.3 a	9.4 ab	17.9 a
HW1	1.9 b	10.4 a	4.2 ab	14.7 a	16.6 a
HW3	2.3 b	6.3 ab	2.1 abc	8.4 ab	10.7 b
HW4	4.3 ab	3.9 abc	2.9 ab	6.8 ab	11.2 b

1. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

Two varieties of the pathogen were responsible for recent outbreaks, *V. fungicola* var. *fungicola* in Europe and var. *aleophilum* in USA and Canada. Previous experiments (Juarez del Carmen *et al.*, 2002) carried out in small closed cells to avoid dissemination of spores, have shown that the var. *fungicola* isolate VCTC produced less numerous bubbles than the var. *aleophilum* isolate V-35. Breeding for resistance to *V. fungicola* implies to identify *A. bisporus* strains resistant or highly tolerant to both varieties. It was with this aim that cultivars and wild strains were compared at PPO MRU facilities for their susceptibility to three var. *aleophilum* isolates collected during the 1999 outbreak in Pennsylvania and two var. *fungicola* isolates responsible for the disease in The Netherlands.

Significant differences in *A. bisporus* susceptibility and *V. fungicola* aggressiveness were detected by analysis of variance, and no significant interaction occurred between *A. bisporus* strains and *V. fungicola* isolates (Table 6). Consequently, percentages of affected mushrooms shown on Table 7 are means of data from the three cell-tests.

Table 6
Analysis of variance for the various symptoms

Variable	*Source*	*df*	*Mean square*	*F value*
S	*A. bisporus*	8	223.08	12.96 **
	V. fungicola	5	319.63	18.57 **
	*A. bisporus * V. fungicola*	40	19.11	1.11 ns
	Cell test	2	10.03	0.58 ns
BO + DB	*A. bisporus*	8	678.89	26.17 **
	V. fungicola	5	339.48	13.09 **
	*A. bisporus * V. fungicola*	40	19.53	0.75 ns
	Cell test	2	108.45	4.18 *
S + BO + DB	*A. bisporus*	8	769.13	36.01 **
	V. fungicola	5	699.82	32.76 **
	*A. bisporus * V. fungicola*	40	24.37	1.14 ns
	Cell test	2	60.62	2.84 ns

** = significant at $P < 0.01$, ns = not significant at $P = 0.05$.

Table 7
Effect of the various isolates of *V. fungicola* on the susceptibility of *A. bisporus*

Treatment		*Percentages of*[1]		
		S	*BO + DB*	*S + BO + DB*
var. *aleophilum*	V02	3.97 a[2]	7.2 a	11.2 a
	V07	4.39 a	7.4 a	11.8 a
	V17	4.25 a	6.0 a	10.3 a
var. *fungicola*	V010404	1.31 b	5.5 a	6.8 b
	V9503	0.69 bc	3.2 b	3.9 c
Control		0.37 c	1.1 b	1.5 d

1. Mean percentages for all the *A. bisporus* strains.
2. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

Table 8
Comparison of the susceptibility of the *A. bisporus* strains to both varieties of *V. fungicola*

A. bisporus	*% S*		*% (BO + DB)*		*% (S+BO + DB)*	
	/ var. fung.	*/ var.* aleo	*/ var.* fung.	*/ var.* aleo	*/ var.* fung.	*/ var.* aleo
A15	0.5 b[1]	6.4 a	13.5 a	17.1 a	14.0 b	23.5 a
U1	2.1 b	9.8 a	12.9 a	17.4 a	14.9 b	27.2 a
W1	0.1 a	1.0 a	2.3 a	3.4 a	2.4 a	4.4 a
W2	1.0 a	2.6 a	0.5 a	1.1 a	1.5 a	3.6 a
W3	1.9 a	2.4 a	1.1 a	3.2 a	3.0 a	5.5 a
W4	1.6 b	8.7 a	2.7 a	3.0 a	4.3 b	11.7 a
W5	0.7 b	2.5 a	2.6 a	1.0 a	3.3 a	3.5 a
W7	0.7 b	3.4 a	2.5 a	7.0 a	3.1 b	10.4 a
W8	0.4 a	1.3 a	0.8 a	5.1 a	1.1 a	6.4 a

1. For a same strain and a same symptom values following by the same letter do not differ significantly at $P = 0.05$.

When all the strains of *A. bisporus* were taken as a whole, they were more susceptible to the three var. *aleophilum* isolates than to the two var. *fungicola* isolates (Table 7).

Looking at the strains individually showed that all, except W5, were more susceptible to var. *aleophilum* than to var. *fungicola* isolates. The low percentages of affected mushrooms produced by W1, W2, W3 and W8 may explain why the differences in susceptibility related to the variety of pathogen were not significant. The percentage of bubbles was slightly but not significantly higher after contamination with var. *aleophilum* isolates. The difference in *A. bisporus* susceptibility to both varieties was mainly related to the propension of the var. *aleophilum* isolates to induce a greater production of spotted mushrooms. Considering the high aggressiveness of the *aleophilum* variety, wild strains highly tolerant to *V. fungicola* were detected in the PPO MRU collection (Table 8).

4. Conclusion

Strains of *A. bisporus* very tolerant to *V. fungicola* exist and can be introduced in breeding programmes but this work shows the importance of the use of

several *V. fungicola* isolates to assess the level of tolerance of future hybrids. Even the differences in susceptibility of the more tolerant strains to both varieties of the pathogen were not significant, the resistance of the selected hybrids to var. *aleophilum* isolates would be determined.

5. References

Collopy P.D., Largeteau-Mamoun M.L., Romaine C.P. and Royse D.J., 2001. Molecular phylogenetic analyses of *Verticillium fungicola* and related species causing dry bubble disease of the cultivated button mushroom, *Agaricus bisporus*. Phytopathology 91: 905-912.

Juarez del Carmen S., Largeteau-Mamoun M.L., Rousseau T., Regnault-Roger C. and Savoie J.M., 2002. Genetic and physiological variation in isolates of *Verticillium fungicola* causing dry bubble disease of the cultivated button mushroom *Agaricus bisporus*. Mycol. Res. 106: 1163-1170.

Kerrigan RW., 1996. Characteristics of a large collection of edible wild mushroom germ plasm: the *Agaricus* Resource Program, pp. 302-307 in Samson, R. A. et al., eds. Culture Collections to Improve the Quality of Life. Centraalbureau voor Schimmelcultures, P.O. Box 273, 3740 AG Baarn, The Netherlands.

Largeteau M.L., Rodier A., Rousseau T., Juarez del Carmen S., Védie R. and Savoie J.M., 2004. *Agaricus bisporus* susceptibility to *Verticillium fungicola*. In. Science and Cultivation of Edible and Medicinal Fungi (Romaine, Keil, Rinker & Royse, eds.), pp. 515-523. Pennsylvania State University.

North L.H. and Wuest P.J., 1993. The infection process and symptom expression of verticillium disease of *Agaricus bisporus*. Can. J; Plant Pathol. 15: 74-80.

Rinker D. and Wuest P.J., 1994. Pest of commercial mushroom production: fungal diseases. Mushroom World, March 1994: 2525-26, 28-29, 32-34.

Sonnenberg A.S.M., Baars J.J.P., Hendrickx P.M. and Kerrigan R.W., 2005. Breeding mushrooms: state of the art. Acta Edulis Fungi 12 (Supp.) 163-173.

Van der Geijn J., 1982. Fungal diseases: practice. Mushroom J. 113: 157-160.